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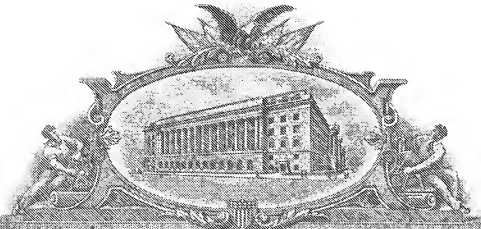

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APPLICATION NUMBER: ***60/548,509***
FILING DATE: ***February 26, 2004***
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Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
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Attorney Docket No. 60805-P (49163)
PATENT

Preliminary Classification:
Proposed Class:
Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. Section 601, 7th ed.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: David Alan Zacharias

For: FUNCTIONAL GENOMICS AND GENE TRAPPING IN HAPLOID CELLS

Mail Stop: Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

COVER SHEET FOR FILING PROVISIONAL APPLICATION (37 C.F.R. SECTION 1.51(c)(1))

CERTIFICATION UNDER 37 C.F.R. SECTION 1.10*
*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on February 26, 2004 (date), in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. Section 1.10, Mailing Label Number EV 438 991 028 US addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Jennifer K. Rosenfield (Reg. No. 53,531)
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. Section 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

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WARNING: "A provisional application must also include the cover sheet required by Section 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this Section." 37 C.F.R. Section 1.53(c)(1). See also M.P.E.P. Section 201.04(b), 6th ed., rev. 3.

NOTE: "A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application." Notice of December 5, 1994, 59 Fed. Reg. 63,951, at 63,953.

"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

NOTE: "A provisional application is not entitled to the right of priority under 35 U.S.C. Section 119 or 365(a) or Section 1.55, or to the benefit of an earlier filing date under 35 U.S.C. Section 120, 121 or 365(c) or Section 1.78 of any other application. No claim for priority under Section 1.78(a)(3) may be made in a design application based on a provisional application. No request under Section 1.293 for a statutory invention registration may be filed in a provisional application. The requirements of Sections 1.821 through 1.825 regarding application disclosures containing nucleotide and/or amino acid sequences are not mandatory for provisional applications." 37 C.F.R. Section 1.53(c)(3).

NOTE: "No information disclosure statement may be filed in a provisional application." 37 C.F.R. Section 1.51(d). "Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 Fed. Reg. 63,591, at 63,594.

NOTE: "No amendment other than to make the provisional application comply with the patent statute and all applicable regulations, may be made to the provisional application after the filing date of the provisional application. " 37 C.F.R. Section 1.53(c).

NOTE: 35 U.S.C. 119(e) provides that "[i]f the day that is 12 months after the filing date of a provisional application falls on a Saturday, Sunday, or Federal Holiday within the District of Columbia, the period of pendency of the provisional application shall be extended to the next succeeding secular or business day."

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. Section 1.51(c)(1)(i). The following comprises the information required by 37 C.F.R. Section 1.51(c)(1):

1. The name(s) of the inventor(s) is/are (37 C.F.R. Section 1.51(c)(1)(ii)):

David Alan Zacharias

NOTE: "If the correct inventor or inventors are not named on filing, a provisional application without a cover sheet under Section 1.51(c)(1), the later submission of a cover sheet under Section 1.51(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship." 37 C.F.R. Section 1.48(f)(2).

NOTE: "The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in Section 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(a)[,] application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that Section 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C. 111(a) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111(a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

See 37 C.F.R. Section 1.53.

	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME
1.	David	Alan	Zacharias
2.			
3.			

2. Residence address(es) of the inventor(s), as numbered above (37 C.F.R. Section 1.51(c)(1)(iii)):

1. 20 Lakeside Place West, Palm Coast, FL 32176
2.
3.

3. The title of the invention is (37 C.F.R. Section 1.51(c)(1)(iv)):
FUNCTIONAL GENOMICS AND GENE TRAPPING IN HAPLOID CELLS

4. The name, registration, customer and telephone numbers of the practitioner *(if applicable)* are (37 C.F.R. Section 1.51(c)(1)(v)):

Name of practitioner: Jennifer K. Rosenfield

Reg. No. 53,531 Tel. (617) 439-4444

Customer No. 21874

(complete the following, if applicable)

[ ] A power of attorney accompanies this cover sheet.

5. The docket number used to identify this application is (37 C.F.R. Section 1.51(c)(1)(vi)):

Docket No. 60805-P (49163)

6. The correspondence address for this application is (37 C.F.R. Section 1.51(c)(1)(vii)):
Jennifer K. Rosenfield
Edwards & Angell, LLP, P.O. Box 55874, Boston, MA 02205

7. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. Section 1.51(c)(1)(viii)).

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

[X] No
[ ] Yes

The name of the U.S. Government agency and the Government contract number are:

8. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. Section 1.51(c)(2)-(3):

Specification: 36 pages
Drawings: 4 sheets (Figures 1-4)

B. Additional documents:

[X] Claims: 1 page
[X] Abstract: 1 page

Note: See 37 C.F.R. Section 1.51.

[ ] Power of attorney
[ ] Small entity statement
[ ] Assignment

NOTE: Provisional applications may be filed in a language other than English as set forth in existing Section 1.52(d). However, an English language translation is necessary for security screening purposes. Therefore, the PTO will require the English language translation and payment of the fee mandated in Section 1.52(d) in the provisional application. Failure to timely submit the translation in response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 111(a) application is filed without providing the English language translation in the provisional application, the English language translation will be required to be supplied in every 35 U.S.C. 111(a) application claiming priority of the non-English language provisional application. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

9. Fee

The filing fee for this provisional application, as set in 37 C.F.R. Section 1.16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

[X] Applicant is a small entity.
[ ] Applicant is not a small entity.

NOTE: "A . . . statement in compliance with existing Section 1.27 is required to be filed in each provisional application in which it is desired to pay reduced fees." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,197.

10. Small entity statement

[ ] The statement(s) that this is a filing by a small entity under 37 C.F.R. Sections 1.9 and 1.27 is(are) attached.

11. Fee payment

[X] Fee payment in the amount of $ 80.00 is being made at this time.

[ ] No filing fee is to be paid at this time. (This and the surcharge required by 37 C.F.R. Section 1.16(l) can be paid subsequently.)

12. Method of fee payment

[X] Check in the amount of $ 80.00.
[ ] Charge Account No. _____, in the amount of $ _____.
A duplicate of this Cover Sheet is attached.

[X] Please charge Account No. 04-1105 for any fee deficiency.

Date: February 26, 2004

Signature of practitioner

Reg. No.: 53,531

Jennifer K. Rosenfield
(type or print name of practitioner)

Tel.: (617 439-4444)

Edwards & Angell, LLP
P.O. Box 55874
Boston, MA 02205
P.O. Address

Customer No.: 21874

BOS2_435317.1

FUNCTIONAL GENOMICS AND GENE TRAPPING IN HAPLOID CELLS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention features methods and compositions for performing functional genomics and gene trapping using haploid cells, including haploid vertebrate cells. The present invention further provides methods for identifying genes involved in cellular signaling pathways.

2. Background

Gene trapping or random insertional mutagenesis is a method used to discover genes responsible for a particular phenotypic characteristic of an organism. Traditionally a mutagenic element, sometimes also containing a reporter element, is introduced in a stochastic and random way into the genome of embryonic stem (ES) cells by means of a viral vector and/or electroporation. The randomly-mutagenized ES cell lines are characterized and then possibly selected on the basis of some morphological, biochemical or other criterion then injected into blastocysts, which are implanted into females and go on to form chimaeric animals. Animal lines harboring the mutation of interest in the germline tissue are then bred to homozygosity and the resulting phenotype studied in the whole, mutant animal, or in some tissue or cell of interest taken from the mutant animal.

The process of generating mutant animals in this fashion is labor and cost intensive to the point of being prohibitive for many research facilities. Likewise, the time involved is also substantial requiring many months before experiments on the "gene-trapped" animal can begin. The motivation to use such a system is that most commonly used cell lines are diploid and as such insertion of a mutagenic element will at most probably hit only one of two alleles of a gene present in the genome. It is unsafe to assume and indeed very unlikely that inactivation of a single allele will be sufficient to eliminate totally the function of a particular gene thereby necessitating elimination of both alleles of a diploid cell line.

Accordingly, there is a need in the art for high-throughput screening methods which allow the use of gene-trapping and functional genomics but do not require the generation of live animals.

SUMMARY OF THE INVENTION

Performing gene-trapping (e.g., insertional mutagenesis) and functional genomics methods in diploid cells is impractical because of the near-impossibility of knocking out both copies of any particular gene. Alternatively, if one applies insertional mutagenesis to a cell line that is haploid, mutation and inactivation of any single gene should result in elimination of the function of that gene as there is only a single copy of the gene represented in the haploid cell line. The introduction of the mutagenic element can occur via a variety of mechanisms with retrovirus and electroporation are the most common means. A stable haploid cell line exists and is available commercially from ATCC (Accession No. CCL-145). This cell line is fibroblast-like, and adherent, two properties that make it useful for microscopic imaging. These cells provide the necessary genomic composition to carry out functional genomics experiments such as random insertional mutagenesis using high-throughput/high content microscopy (HCM). Additionally, it is possible to develop independently a haploid line from another animal including mouse and potentially human. Following mutagenesis, cellular morphological or physiological readouts selected to identify specific genes that alter the morphology or physiology of interest can be carried out using HCM.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts various lipid modifications : A. prenylation and B. Acylation. Each class of modification targets proteins to which they are attached to unique subcellular locales (Melkonian et al., 1999; Moffett et al., 2000; Zacharias et al., 2002). This ability is likely due to their varying chain length, degree of saturation and their physical position on the proteins. Both forms of prenylation occur via stable thioether

bonds on the final cysteine of a "CAAX" box at the C-terminus of a protein. Myristoylation occurs via a stable amide bond to the N-terminal glycine of a protein while addition of palmitate occurs most commonly via a labile thioester bond to the side chain of a free, reactive cysteine on the cytoplasmic side of the PM.

Figure 2. Epifluorescence micrographs of transiently-expressed A. GAP-43:GFP and B. GFP. Expression of GAP-43:GFP is localized heavily on the PM of MDCK cells **A**. A 10-residue peptide from the N-terminus of GAP-43, fused to GFP is doubly palmitoylated. This post-translational modification is sufficient, in and of itself, to direct the sensor to the PM as compared to the GFP alone which is evenly distributed throughout the cells **B**. No other targeting signal is known to exist within the 10 residues (NH2-MLCCMRRTKQ) fused to GFP other than the two cysteines (underlined) which are palmitoylated.

Figure 3. A Schematic representation of the components used for insertional mutagenesis. The structure and arrangement of the components are similar whether electroporating plasmid DNA or infecting by retrovirus except that the retroviral constructs will contain long terminal repeat (LTR) sequences flanking the reporter cDNAs. SA, Splice acceptor (5'GTCCCAGGTCCCGAAAA- from the mouse engrailed 2 gene); GFP, Green Fluorescent Protein; pA, polyadenylation sequence that will follow a series of stop codons (XXX) in all three reading frames; RSV, a promoter to drive expression of neo, a neomycin resistance gene/protein to enable selection of stable integrants by virtue of their antibiotic resistance. A consensus splice donor site sequence (5'-CCG CTC GAG ACT TAC CTG ACT GGC CGT CGT TTT AA GAC GAG CTC CCT AGC TAG TCA GGC ACC GGG CTT-(Zambrowicz et al., 1998)) SD to ensure proper splicing with a downstream exon/poly-adenylation site. The three lines represent (| | |) palmitoyl groups that will localize the fusion protein to the PM. The asterisk (*) indicates the point of random fusions. Expression of GFP:SPS is driven by the promoter from the trapped gene.

Figure 4. Potential outcomes when "trapping" genes in a diploid or haploid cell line. Plasma membrane (PM) localization of an S-palmitoylation substrate (SPS) fused to GFP (GFP:SPS), **a.** Functionally disrupting " "a single allele of a critical gene in a diploid cell line could have no apparent visual affect on the PM localization of an

GFP:SPS **b**; it could have a partial affect **c**, displacing a variable amount of GFP:SPS from the PM; a convenient result would be complete displacement of GFP:SPS from the PM to the cytoplasm by mutagenizing a single allele in a diploid cell **d**. The likelihood of displacing a significant fraction of GFP:SPS form the PM may be increased by using a **haploid** (frog, *Rana*) cell line **e-g**. PM localization of GFP:SPS **e**. Mutagenizing (functionally disrupting) a single allele of a cirtical gene in such a line would increase the likelihood of inducing a completely cytosolic localization **f**, except in the case where functional redundancy among members of a gene family can restore partial function **g**. The functional redundancy problem would be true in the diploid cell line as well.

DETAILED DESCRIPTION OF THE INVENTION

Performing gene-trapping (e.g., insertional mutagenesis) and functional genomics methods in diploid cells is impractical because of the near-impossibility of knocking out both copies of any particular gene. Alternatively, if one applies insertional mutagenesis to a cell line that is haploid, mutation and inactivation of any single gene should result in elimination of the function of that gene as there is only a single copy of the gene represented in the haploid cell line. The introduction of the mutagenic element can occur via a variety of mechanisms with retrovirus and electroporation are the most common means. A stable haploid cell line exists and is available commercially from ATCC (Accession No. CCL-145). This cell line is fibroblast-like, and adherent, two properties that make it useful for microscopic imaging. These cells provide the necessary genomic composition to carry out functional genomics experiments such as random insertional mutagenesis using high-throughput/high content microscopy (HCM). Additionally, it is possible to develop independently a haploid line from another animal including mouse and potentially human. Following mutagenesis, cellular morphological or physiological readouts selected to identify specific genes that alter the morphology or physiology of interest can be carried out using HCM. Such microscopy platforms pioneered by Cellomics Inc. have been under development for use in drug discovery for the last 6-8 years.

Recent advances in machine visions have resulted in an explosion of the application of imaging to cell biology. Several companies including Q3DM (San Diego,

CA) have developed microscopic platforms and machine vision algorithms with a degree of sophistication that they are being implemented by major pharmaceutical companies for mainstream drug discovery and by academic labs with a need for extremely quantitative analyses of morphological events in cell biology. The combination of functional genomics like gene trapping and HCM has tremendous untapped potential especially in academia.

An example shows how useful the combination of a haploid cell line and HCM is (see also attached figure 1): It is possible to determine the enzymatic cascade of genes responsible for the various forms of protein palmitoylation, a post-translational modification of proteins that causes the host protein to be associated with a cellular membrane, commonly the plasma membrane. Briefly, this is accomplished by creating a stable, haploid line expressing Green Fluorescent Protein (GFP) genetically fused to a short peptide substrate (S-palmitoylation substrate: SPS—in this case) for palmitoylation or any lipidation reaction of interest. This stable cell line would then be randomly mutagenized, or "gene trapped" using a standard mutagenic element and procedures. Selective pressure in the form of antibiotic resistance can enrich the cell pool for tens to hundreds of thousands of individual cell lines that harbor both the mutagenic element and the stably expressed GFP:SPS transgene. If the mutagenic element disrupts a gene directly or possibly indirectly responsible for the addition of the lipid adduct to the GFP:SPS substrate the fluorescent reporter of lipidation, GFP:SPS, will no longer be localized to the cellular membrane as it would under normal circumstances. Rather the protein will be localized to the cytoplasm as non-palmitoylated GFP is normally. Determining the genes into which the mutagenic element landed resulting in the redistribution of GFP from the membrane to the cytoplasm is a standard procedure. The task of screening tens of thousands of individual mutated cell lines for a gross change in the distribution of a fluorescent reporter like GFP is made simple by using an HCM system like the EIDAQ100 from Q3DM. Existing algorithms allow for discriminating morphological details such as the redistribution of a fluorescent reporter from the membrane to the cytoplasm and it is capable of discriminating interesting mutants from mutants benign to the morphology of interest with enough speed to completely assay the entire genome three times per day assuming that the genome consists of roughly 30,000

unique genes. This process would take many months of expensive labor if scoring such changes by eye.

The process describe above can be applied easily to any case where there is a change in physiology or morphology and an algorithm exists that can describe and quantitate the event. A large library of metrics exists now making it relatively simple to tailor a system to ones needs. The combination of this emerging technology with the haploid cells is completely unique and has the potential to rapidly elucidate all steps of signaling cascades that regulate processes that can be detected using fluorescence microscopy.

Many proteins are concentrated on the plasma membrane (PM), trapped in specialized subcellular regions, like synapses and caveolae, by virtue of their lipid modifications. Thio-acylation or S-palmitoylation, a common form of lipid modification, is unique in that it is reversible and dynamic, suggesting a modulatory role in signal transduction similar to phosphorylation. Recent data indicate that proper, dynamic regulation of the palmitoylation of PSD-95, an abundant scaffolding protein in the synapse, is critical for synaptic organization and function, linking palmitoylation to complex processes such as learning, memory and disease. In support of this position, it is known that mutations in one gene regulating S-palmitoylation result in a severe neurodegenerative disorder, infantile neuronal ceroid lipofuscinosis or ICNL. Additionally, a candidate gene for the regulation of S-palmitoylation is linked to schizophrenia. These examples highlight the importance of properly regulated S-palmitoylation to human health and disease. Biochemical characterization of the enzymes responsible for S-palmitoylation (palmitoyl thio-acyl transferases, S-PATs) has been difficult and controversial; recent data from experiments in yeast add substantial weight to the argument that such enzymes exist. To date, functional genomics discovery programs in vertebrate systems similar to those in yeast have been expensive and time consuming. Experiments in this proposal address this issue by combining a novel form of gene-trapping in vertebrate cell cultures, with a fully automated readout in a high-throughput microscopy (HTM) format. With this assay system we will be able to test directly and functionally the hypothesis that S-PATs exist in vertebrates. Should this prove to be the case, we will be ideally positioned to elucidate the entire enzymatic

pathway for protein S-palmitoylation by quantitatively analyzing millions of cells from tens of thousands of "trapped" cell lines.

Development of the system described in this proposal, using S-palmitoylation as the proof-of-concept, model pathway will provide critical information about the regulation of S-palmitoylation and an invaluable experimental tool that can be extended to screens for other genes that regulate the subcellular distribution and concentration of proteins enabling numerous applications in basic and therapeutic research. Toward this end, the following two specific aims are proposed:

Many proteins are concentrated on the PM by virtue of their lipid modifications. Recent data show that lipid modifications of proteins may well be the primary physical determinant for targeting to and retention of some proteins to membrane lipid microdomains such as synapses and caveolae (El-Husseini et al., 2000; El-Husseini Ael et al., 2002; Kanaani et al., 2002; Loranger & Linder, 2002; Topinka & Bredt, 1998; Zacharias et al., 2002). Fusion of Green Fluorescent Protein (GFP) to small-peptide substrates for lipid modification (e.g. the N-terminal 10 residues of GAP-43) has been shown to be sufficient to localize the fusion proteins to the PM in the absence of any other targeting signal (Zacharias et al., 2002). Similarly, mutagenic substitution of modifiable residues for ones which cannot be modified results in gross mislocalization and/or loss of function of the expressed proteins (Craven et al., 1999; Hiol et al., 2003; Osterhout et al., 2003; Wiedmer et al., 2003). It has also been shown that different lipid moieties induce partitioning into different lipid environments or lipid microdomains of cells (Melkonian et al., 1999; Moffett et al., 2000; Zacharias et al., 2002). Specific associations of proteins within such microdomains, whether mediated by attractive, protein-protein interactions, forced proximity or both, are critical components in the architecture of cellular communication and lipid modifications undoubtedly play an important role in the creation and modulation of such protein associations. This point is amply demonstrated by the fact that the regulation of protein lipidation is known to be involved in several forms of cancer (Dinsmore & Bell, 2003; Ghobrial & Adjei, 2002), a severe neurodegenerative disorder, ICNL (Vesa et al., 1995) and possibly for schizophrenia (Liu et al., 2002). New optical methods and fluorescent sensors, (like those described in this proposal) for characterizing the state of protein lipidation, protein

localization and protein interactions are consistently at the technological forefront, driving new discoveries in these fields and providing insight into the intricate, structured workings within cells and in networks of cells (Lippincott-Schwartz & Patterson, 2003; Weijer, 2003; Zacharias et al., 2000).

Prenyl and Acyl groups are the most common forms of protein lipid modifications (Figure 1). The two most common forms of prenylation are geranylgeranylation and farnesylation (Figure 1A) while myristoylation and palmitoylation (Figure 1B) are likely the most common forms of acylation. Most, if not all of the biochemical steps regulating the prenylation of proteins have been deciphered (reviewed in (Fu & Casey, 1999; Roskoski, 2003; Sinensky, 2000)). In fact, the mechanistic pathway for farnesylation has been determined to the atomic level (Long et al., 2002). This density of information for prenylation is due in part to the fact that prenyltransferases have been fairly successful therapeutic targets for the treatment of several types of cancer (Dinsmore & Bell, 2003; Ghobrial & Adjei, 2002), underscoring the importance of protein lipidation for human health and disease.

In contrast to prenylation, a detailed knowledge about the process of protein acylation is still lacking. Among the types of acylation, enzymatic processes regulating myristoylation have been characterized best and are reviewed in (Farazi et al., 2001; Rajala et al., 2000; Resh, 1999) Briefly, proteins that will become myristoylated begin with a consensus sequence Met-Gly-X-X-X-Ser/Thr. The start Met is co-translationally, proteolytically removed and the myristate is added to the exposed N-terminal glycine via a stable amide bond. The formation of this bond is catalyzed by N-myristoyl transferase with a high degree of selectivity for 14-carbon myristate (Farazi et al., 2001; Rajala et al., 2000). N-terminal myristoylation often exists in combination with palmitoylation which can take at least two forms: N-palmitoylation (apparently rare) and S-palmitoylation (the most common). N-palmitoylation, first described for the protein sonic hedgehog (Pepinsky et al., 1998), is the addition of palmitic acid to the a-amide of Cys-24, which is proteolytically exposed to become the N-terminal residue of the functional protein. Addition of a palmitoyl group by an amide bond to the N-terminal glycine was recently shown to occur on the heterotrimeric G-protein, Gas (Kleuss & Krause, 2003). S-palmitoylation, the focus of this proposal, is a reversible modification that occurs via a

labile thioester bond with the thiol side chains of reactive cysteine residues on the cytoplasmic portions of a protein. Proteins that are palmitoylated are relatively abundant and diverse (Melkonian et al., 1999; Moffett et al., 2000) and the functional consequences of the modification vary depending on the protein. But in general, palmitoylation increases the hydrophobicity of a protein thereby affecting the degree of membrane association as well as sublocalization within a membrane. Once associated with the membrane, the palmitoyl group partitions primarily into cholesterol- and sphingolipid-rich lipid rafts (Moffett et al., 2000; Zacharias et al., 2002). The additional membrane avidity increases the likelihood that the palmitoylated protein will interact (forced proximity) with other membrane-bound or membrane-associated proteins, a phenomenon that is exemplified by the synaptic scaffolding protein, PSD-95(Craven et al., 1999; El-Husseini et al., 2000; Perez & Bredt, 1998; Topinka & Bredt, 1998). The resulting complexes of interacting proteins constructed at sites of membrane specialization like synapses are critical nodes for signaling containing proteins that are involved in every conceivable signaling pathway. Furthermore, modulation of the associative properties of individual proteins in these networks by reversibly palmitoylating some members, is a very attractive and plausible mechanism to regulate the participation of proteins in signaling events (el-Husseini Ael & Bredt, 2002; El-Husseini Ael et al., 2002; Hess et al., 1993; Qanbar & Bouvier, 2003).

The finding that the residence half life of the palmitoyl group on proteins is considerably shorter than the half life of the proteins to which it is attached (Lane & Liu, 1997; Wolven et al., 1997) suggested that enzymes for palmitate removal, protein palmitoylthioesterases or PPTases, could exist. PPT1 (Camp & Hofmann, 1993; Camp et al., 1994) is a lysosomal hydrolase that participates in the degradation of palmitoylated proteins by deacylating cysteine thioesters; acyl protein thioesterase 1 (APT1), a cytoplasmic protein first biochemically characterized as an acyl thioesterase by Duncan and Gilman (1998), is a member of the serine hydrolase, α/β fold family of lysophospholipases that has several additional substrate and lipid specificities (Duncan & Gilman, 1998). Regulated removal of the palmitoyl group from proteins is critical in human health as defects in PPT1 result in a severe neurodegenerative disorder known as infantile neuronal ceroid lipofuscinosis (ICNL) (Vesa et al., 1995). The role of PPT1 in

ICNL was confirmed by targeted disruption of the gene in a mouse resulting in a model of the human disorder (Gupta et al., 2001).

Elucidation of the regulation of protein S-palmitoylation has lagged significantly behind, and has been more controversial than for depalmitoylation. Reconstruction of the enzymatic pathway that leads to protein palmitoylation using novel methods is the primary focus of this proposal. There is currently no known amino acid consensus sequence for palmitoylation, suggesting either 1) a non enzymatic mechanism for palmitoylation or equally as likely 2) the existence of multiple, unidentified enzymes whose homo/hetero stoichiometry, cofactors or other undefined factors are unknown. Palmitoyl-CoA can spontaneously acylate cysteines residues of some fragments of full length proteins that are normally palmitoylated (Bharadwaj & Bizzozero, 1995; Quesnel & Silvius, 1994) and some, but not all, fully-folded, normally-acylated proteins.(Duncan & Gilman, 1996). For example, myristoylated $G_{i\alpha 1}$ is efficiently and stoichiometrically auto-palmitoylated on Cys^3 (Duncan & Gilman, 1996). However, in the same study, SNAP-25, GAP-43 and Fyn kinase were not spontaneously acylated. Since the mid 1980s, biochemists have been able to purify, to varying degrees, S-PAT activity from different cell and membrane types (Berger & Schmidt, 1984; Berthiaume & Resh, 1995; Dunphy et al., 1996; Mack et al., 1987). The biochemical characterization of these proteins has been difficult and has, in some cases, led to the discovery of proteins that were involved in lipid metabolism rather than the transfer of palmitoyl groups. Using genetic approaches in yeast, two proteins with S-PAT activity have been identified and characterized: Erf2p (Bartels et al., 1999; Dong et al., 2003; Long et al., 2002) and Akr1p (Roth et al., 2002) (reviewed by Linder and Deschenes (2003)). The Erfp2 coding sequence predicts four transmembrane spanning domains and Arkp1, six transmembrane spanning domains. Both proteins contain DHHC-CRD domains (i.e., Asp-His-His-Cys-Cysteine Rich Domain) (Putilina et al., 1999) thought to be responsible for the S-PAT activity as mutations in the DHHC domain abolish S-PAT activity(Bartels et al., 1999) and because homology between these two proteins, both with S-PAT activity, is limited to the DHHC-CRD domains. Orthologous proteins exist in every eukaryote examined so far (Linder & Deschenes, 2003) emphasizing the fundamental importance of S-palmitoylation. It is also important to note that a protein containing a DHHC-CRD motif

and highly homologous to Erf2p was identified as a candidate gene for schizophrenia (Liu et al., 2002). Whether the candidate gene encodes an S-PAT remains to be seen, but if so it raises the possibility that pharmacologically modulating S-PAT activity in the brain could result in a treatment for schizophrenia. Development of the concepts and methods described in this proposal would form the basis for a successful high-throughput screen in a search for small-molecule modulators of S-PAT activity.

Functional Genomics and High Throughput Microscopy as Methods for Studying Signal Transduction

Human orthologs of the yeast proteins have been identified from database queries but whether all DHHC-CRD proteins exhibit S-PAT activity or whether they act alone, as hetero/homo-oligomers, or in concert with cofactors or other proteins remains to be seen. Efforts to clone and characterize all of the DHHC-CRD proteins in search of S-PAT in mammalian systems are surely underway by a number of groups including those who first identified them in yeast. The first successful identification of these proteins took advantage of the genetic tractability of yeast. Similar genetic manipulations in vertebrate systems such as mice are far more labor intensive but often times required, for example when the mutant phenotype affects the development of the organism. However, when a mutation is expected to cause a visually identifiable change in the concentration or distribution of a protein, it becomes possible to reduce the experimental model to one where the gene of interest is mutated in a cultured cell line and the phenotype analyzed using microscopy. Specifically, using gene- and/or promoter-trap mutagenesis and examining the resulting mutant cell lines using HTM, would enable cell-based experimental analysis of gene function, on a genome-wide scale. This nascent technology can rapidly (on the order of hours) screen tens of thousands of mutated, stable cell lines covering millions of cells, for one or more mutants having a phenotype that is indicative of a gene that is directly, or indirectly, involved in regulating protein distribution or concentration. In the proposed case the proteins would be those involved in the addition of palmitates to a protein(s) or peptide responsible for the biosynthesis of palmitate, palmitate-CoA. The morphological metric of interest in this case will be the subcellular localization of the reporter, Green Fluorescent Protein (GFP) fused to an S-

palmitoylation substrate (SPS); GFP:SPS. Briefly, under normal circumstances GFP:SPS would be localized at the PM. However, if the reporter construct mutates a gene in the signaling pathway for S-palmitoylation, GFP:SPS will relocalize from the PM to the cytoplasm.

Gene trapping as a method to identify S-PATs

Gene- and promoter-trapping are forms of insertional mutagenesis whereby reporter genes and/or selectable markers are randomly and likely stochastically (Chowdhury et al., 1997; Evans, 1998) inserted into the genome of mouse ES cells (reviewed in: Cecconi & Meyer, 2000; Cecconi & Gruss, 2002; Stanford et al., 2001). Traditionally functional genomics studies employing gene-trap have relied on "trapping" the genes in ES cells, generating lines of mice with the mutated ES cell lines, then analyzing the phenotype resulting from the mutation in the whole animal (Evans et al., 1997; Stanford et al., 2001). This approach, while very informative and necessary in many cases (such as the role of the gene in the development of the animal), is labor intensive and expensive. The proposed experiments do not seek to circumvent or replace the process of screening mutant phenotypes in mice when it is necessary. Rather, the development of the proposed method will 1) provide a screening format that will enable the dissection of signaling pathways at the cellular level using gene-trap technology 2) provide an enabling technological platform wherein the prescreening of ES cells for a desired mutation will enhance the process of generating mutant mice by pre-selecting mutant ES cells displaying a desired or predictive, characteristic phenotype.

To achieve the daunting goal of screening thousands of cell lines to determine the subcellular localization of the GFP:SPS reporter, I propose adapting HTM (also referred to as high-content screening or HCS) to the functional genomics format. HTM is a technology just now coming of age in drug discovery research (Milligan, 2003; Price et al., 2002; Woollacott & Simpson, 2001) in pharmaceutical companies. There is a vast and untapped potential for this technology in the academic sector stemming primarily from the fact that this platform provides the most quantitative mechanism for doing cell biological experiments of virtually any type (Price et al., 2002). Traditionally, the method for determining subcellular localization of proteins has been visual inspection and

interpretation of relatively low numbers of microscopic images of cells expressing a GFP-tagged protein or fluorescent-labeled antibodies with specificity to a protein (Giuliano & Taylor, 1998; Zacharias et al., 2000). However, such subjective interpretations may be unconsciously or consciously influenced by investigator bias. Data obtained this way are not always easily confirmed in independent investigations, are not usually amenable to statistical analysis and are labor intensive. A primary motivating factor in the development of HTM has been the perceived need of pharmaceutical companies (Taylor et al., 2001) to investigate rapidly the effects of millions of small molecule compounds on a target molecule within the complex environment of a living cell; an environment where entire, endogenous intracellular signaling pathways are hopefully intact and functional. It has been the hope of the developers of HTM and the pharmaceutical industry that the increased information density that can be generated using multiplexed fluorescent readouts of drug responses would increase the quality of "hits" in a primary (> a million compounds) screen as well as decrease the time required to determine if lead compounds were functioning via the desired or predicted signaling routes. While it is likely that this type of screening method will prove very useful to the pharmaceutical industry, the instrumentation and machine-vision algorithms that have been developed should also find widespread use in basic, academic research (Yarrow et al., 2003). HTM will add a badly needed quantitative edge to cell biology.

Morphometric Analysis with HTM

Existing HTM algorithms are capable of discriminating minute changes in cell morphology or subcellular protein distribution (Conway et al., 2001; Ghosh et al., 2000; Minguez et al., 2002). There is essentially no limitation on the types of morphology that can be used, alone or combinatorially, as the criteria for a unique marker (Boland & Murphy, 2001; Price et al., 1996; Price et al., 2002; Roques & Murphy, 2002). Multiple criteria ranging from the location or concentration of a fluorophore in a cell(Boland et al., 1998; Boland & Murphy, 1999 a,b;2001; Markey et al., 1999; Murphy et al., 2000; Price et al., 2002; Roques & Murphy, 2002) to a physical change in the shape of a cell or a redistribution of cellular contents such as chromosomes, transcription factors (Ding et al., 1998)microtubules (Minguez et al., 2002) membrane protrusions (e.g. neurites, ruffles

etc)((Price et al., 1996; Roques & Murphy, 2002)) can be used individually or combined to enhance the sensitivity and accuracy (Boland & Murphy, 2001; Price et al., 1996; Roques & Murphy, 2002). These algorithms provide a flexible and unbiased determination of the existence degree of an interesting, visible change in cells allowing for high-resolution determinations of pharmacological efficacy tied to such a change (Conway et al., 2001; Ding et al., 1998; Ghosh et al., 2000). It is the exquisite sensitivity for subtle change and the objectivity of HTM that should make possible the use of cell lines for a wider variety of gene trap screens.

The overall goal of the project will be to design and test a cell-based, high-throughput, functional genomics assay that will identify genes/enzymes comprising the pathway leading to S-palmitoylation of multiple S-palmitoylation substrates. Accomplishing this will provide a substantial foundation for expanding the use of HTM in cell-based, functional genomics in that it will be the basis upon which the development of other changes in cell morphology or fluorescent probe (re)distribution can be applied to gene-trap and functional genomics. It will also provide a conceptual and technical basis for developing a drug discovery screen for small-molecule modulators of protein S-palmitoylation.

Insertional mutagenesis, or gene trap, is normally performed in embryonic stem (ES) cells which are subsequently used to create mutant mouse lines in which the resulting phenotypes are analyzed. We are proposing a novel, complementary format for screening interesting mutations in cell lines using fluorescent reporters of protein localization. We will develop gene trap vectors that will enable the discovery of genes involved in regulating protein S-palmitoylation. We also propose experiments for functional characterization of mutant cell lines identified in our high-throughput screen.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the sequence listing and the figures, are incorporated herein by reference.

EXAMPLES

EXAMPLE 1:

C. Preliminary studies

The basic concepts that are the foundation of this proposal rest on the ability of palmitoylation alone to cause the Green Fluorescent Protein: S-Palmitoylation Substrate or GFP:SPS to be localized to the PM. Having this ability allows us to separate cleanly the role of palmitoylation from the influence of other signals, like protein-protein interactions, that might impinge on subcellular targeting. Though a case of purely isolated S-palmitoylation may not exist naturally, creating it with the reporters described here allows us to look specifically and exclusively for modulators of S-palmitoylation. Quantitatively analyzing images of millions of cells expressing such specific reporters is the second aim of this proposal. Accurately quantifying the amount of fluorophore localized to the PM and cytoplasm requires that the image be segmented. In this case, pixels from the cell image must be correctly identified and categorized into discrete, PM and cytoplasm classes allowing accurate quantitation of the fluorescent reporter at each location. Subsequent analysis of the segmented data will determine the degree of translocation from the PM to the cytoplasm.

Palmitoylation alone is sufficient to cause a protein to associate with the PM

There are many cases in which multiple targeting signals within a full-length protein, work in concert to determine its final, subcellular location. Often, protein-protein interaction domains such as PDZ domains are found in close, linear and/or structural proximity to palmitoylatable cysteines of the same protein. A prime example of this is PSD-95(Craven et al., 1999; El-Husseini et al., 2000; Topinka & Bredt, 1998). Isolating the portion of such a protein that is acting as the SPS is an ideal way to create a sensor specific for palmitoylation rather than for the function of the original, full-length protein from which the peptide was borrowed.

The N-terminus of GAP-43 is doubly palmitoylated. When a 10-residue S-palmitoylation substrate peptide from the N-terminus Gap-43 is fused to the N-terminus of GFP, this peptide alone, by virtue of its palmitoylation, is sufficient to cause GFP (in the absence of any other known targeting signal (Zacharias et al., 2002) such as a polybasic domain (Hancock et al., 1990) or an additional lipid type (Prior & Hancock, 2001)) to be localized to the PM (Figure 2A). This point is important in that if this were not so, the potential for disrupting genes not related to palmitoylation, but still causing redistribution from the PM to the cytoplasm, would be much higher. For example, one could imagine that chaperones or transport proteins that might be necessary for the normal trafficking of the original, full-length, palmitoylated protein would, when disrupted, result in redistribution of the reporter from the PM to the cytoplasm. The GFP:SPS chosen for this study will be free of such ancillary signals for PM targeting or any enzymatic activity present in the parent protein.

The fundamental ability to quantitatively recognize GFP localized to the PM has already been achieved by the engineers at Q3DM using methodology described in the next section. Computer-generated demarcation of the PM and the nucleus are demonstrated in Figure 3. While adequate, in its current state, to meet the needs of the proposed experiments, this algorithm has not been optimized to its fullest potential. Therefore successfully completing the experiments outlined in this proposal is not dependent on the development of an algorithm that is theoretically unachievable.

In sum our preliminary data demonstrate:

1. Palmitoylation alone is sufficient to take the fluorescent reporter GFP to the plasma membrane.
2. The S-palmitoylation substrate, when fused to GFP, can report S-PAT activity independent of any other cellular activity.
3. The HTM component of this proposal does not represent an unachievable roadblock to successful completion of all of the goals of the proposal.

D. Research Design and Methods Design and implementation of gene-trapping strategies in which the mutagenic element is also a fluorescent reporter (by virtue of its subcellular localization) of the activity of the trapped gene.

I. Vector design and introduction of DNA into host cell lines

A schematic of the vectors to be used and their resulting protein fusion products is shown below (Figure 3). All vectors will be made using standard molecular biology techniques. The vectors chosen fall into the polyA-trap (Niwa et al., 1993; Salminen et al., 1998; Voss et al., 1998; Zambrowicz et al., 1998) class in which a splice acceptor site (SA) from the engrailed-2 gene immediately precedes the promoterless reporter gene, GFP, fused to a S-palmitoylation substrate (GFP:SPS). The unique sequences provided by the SA and GFP provide primer sites for 5' RACE. This unit is combined with the gene for neomycin resistance under the independent and constitutive control of its own, RSV promoter. A splice donor (SD) (Zambrowicz et al., 1998) at the 3' end of the NeoR gene enables connection to the polyA tail of the trapped gene. The SD also contains stop codons in all three frames (to prevent C-terminal fusions to the NeoR protein) and unique sequence that will facilitate 3'RACE analysis of the trapped gene as well as the increase the structural integrity of the integrated reporters. An additional advantage of this configuration is that G-418 selection should be possible only when the polyA-trap vector integrates upstream of a splice acceptor and a poly-A site of an endogenous gene; intergenic insertions will be eliminated.

Methods for Integrating DNA into the Genome of the Host Cell Line

The two most common methods used to introduce the mutagenic DNA to the genome are electroporation of plasmid DNA (Chowdhury et al., 1997; Wurst & Joyner, 1993) and by virus-mediated (most commonly retrovirus) infection (Friedrich & Soriano, 1991; Zambrowicz et al., 1998). Each method has advantages and disadvantages but a general consensus is developing that a combination of these two methods is required for complete coverage of the genome (Stanford et al., 2001). The plasmid DNA used for electroporation will be based in the promoterless pBluescript (Stratagene) vector and

introduced into cells using the BioRad GenePulser. For retroviral infection, the Pantropic Retroviral Expression System (BD Biosciences Clontech), which efficiently infects mammalian and nonmammalian hosts including amphibians (*Rana* and *Xenopus*) will be used as starting material and modified as illustrated in Figure 3. This system uses VSV-G, an envelope glycoprotein from the vesicular stomatitis virus that is not dependent on a cell surface receptor but rather mediates viral entry through lipid binding and PM fusion.(Emi et al., 1991) A modified version of the vector pLXRN (BD Biosciences Clontech) will be used to express the reporter of localization GFP:SPS as well as the neomycin resistance gene. Following introduction of the mutagenic DNA by either method, clonal lines will be generated by exposing the cells to G-418. G-418-resistant colonies will either be pooled and replated using limiting dilution (a method that will limit the number of clones to approximately one per well of a multiwell plate), or sorted based on their GFP fluorescence by a FACS and plated at a density of clone per well of a multiwell plate. All experiments using retrovirus will take place in the properly equipped and designated Level 2 Biosafety cell culture facility of the PI.

The trapping vectors are designed to serve the essential basic purposes for the proposed studies. 1) they will provide a mechanism to determine whether a cell line has integrated a copy of the reporter (the fluorescence of GFP) and, simultaneously 2) provide a functional indicator, by virtue of its subcellular localization, for whether the reporter has mutagenized a gene necessary for directing the S-palmitoylation of the SPS. Integration of the gene into a locus involved in S-palmitoylation will result in fluorescence being redistributed from the PM to the cytoplasm. Using the mutagenic element as a fluorescent reporter of protein localization is a novel, and broadly applicable component of the research design. Cell lines expressing a cytoplasmic distribution will be candidates for further characterization. Most cell lines should express prominent labeling of the PM (as in Figure 2A; preliminary results) with some background expression on endomembranes, as is common for this type of expression system (Zacharias et al., 2002).

The first set of experiments will utilize gene-trap vectors that include a SA site fused to the 5' end of the reporter construct (figure 3). When a reporter gene is preceded by an SA, the gene must be inserted into an intron to be expressed; this method will not

trap genes without introns. While this group of genes, including olfactory receptors/GPCRs (reviewed in Gentles & Karlin, 1999; Sosinsky et al., 2000), and interferons (Roberts et al., 1998), is relatively smaller than the group with introns (Gentles & Karlin, 1999), this genomic space is important and must also be surveyed. Promoter traps (e.g.,Hicks et al., 1997) are appropriate tools to identify single-exon genes, and will be incorporated into the experimental program as needed following the initial screens using the polyA-trap vectors.

It is possible that the cDNA encoding the reporters could be physically fractured during the integration event (Voss et al., 1998) giving rise to the possibility that one will be integrated independently from the others. For example it could be the case that GFP becomes separated from the SPS resulting in a completely cytoplasmic pattern for fluorescence localization, or in other words, a false-positive result indicating integration into a gene necessary for S-palmitoylation. For this reason, all clonal lines reporting a positive result will be surveyed for expression of an un-fragmented reporter, GFP:SPS. Reverse transcription-PCR and/or PCR of genomic DNA using primers to the 5' end of GFP and the 3' end of the SPS are efficient ways to determine the integrity of the integrated reporter.

II *Selection and Cloning of Appropriate S-palmitoylation Substrates*

S-palmitoylation substrates will be cloned from whole mouse brain mRNA by RT-PCR. Fusions of the SPSs to GFP will be done by PCR and confirmed by DNA sequencing. Analysis of and selection for the appropriate expression pattern as well as the transfection/infection efficiencies of various permutations of the constructs will be made in small scale transfection experiments prior to running a full-scale screen.

The choice of which SPSs to use, and how they will be fused to GFP, will be based on the tolerances of both GFP and SPSs for N- and C-terminal fusions. The fluorescence properties of GFP are unlikely to be perturbed by fusions to either terminus. In the case of the trapping vectors to be used (figure 3), the exact nature of fusions ("protein X" in figure 3) to the N-terminus of GFP cannot be predicted other than to assume that the fusions will be widely variable in their structure and function. The high degree of tolerance of GFP for fusions makes it a safer candidate to host random fusions.

Conversely, certain contextual requirements exist for some peptides to be permissive substrates for S-palmitoylation. Very generally, four classes of substrate exist (Linder & Deschenes, 2003), **a**. transmembrane proteins, **b**. dually acylated proteins, **c**. exclusively palmitoylated cytoplasmic proteins and **d**. mitochondrial proteins. Each class has a different degree of suitability with members of class **c** being the best for proof-of-concept experiments like those in this proposal. A fine example of a class **c** protein with apparently less rigid context or environmental requirements is Yck2p (Robinson et al., 1999; Roth et al., 2002). This protein has been shown to retain plasmalemmal localization when fused to GFP. The first mutagenesis probe I will use will be GFP:Yck2p(C-terminal tail peptide: NH2-KSSKGFFSKLGCC-COOH) as it is functionally and conceptually as simple as the GAP-43 example shown in the preliminary data section (figure 2A) with one important difference, it is a fusion to the C-terminus of GFP where it is not subject to the contextual instability imposed by random fusions as is the case for an N-terminal fusion.

A significant fraction GFP:Yck2p fluorescence should be localized to the PM as is the case for GAP-43 in Figure 2A, other fluorescence should be associated mostly with endomembranes, not in the cytoplasm. Since only a fragment of Yck2p will be used as the SPS, it is expected that it will retain no activity intrinsic to the native protein that could preclude adequate expression levels.

In the unlikely event that the C-terminus of Yck2p does not localize GFP strongly enough to the PM, additional residues (further N-terminal to those already included) from Yck2p will be included in the fusion in an attempt to restore a preferred environment or context for full S-palmitoylation. Alternatively SPS peptides borrowed from the C-terminus of other proteins (e.g. GRK6 (Stoffel et al., 1994) without the PDZ-binding domain, V2R(Sadeghi et al., 1997), bovine rhodopsin (Ovchinnikov Yu et al., 1988) etc.) will be fused to GFP, evaluated for subcellular expression pattern and substituted when appropriate.

III. *Determination of suitable Cell lines*

S-palmitoylation is a highly conserved function, so in that respect, most every cell type and cell line could be used.

Cell lines generated by insertional mutagenesis will be screened using HTM, for 1) fluorescence, indicating that the vector has integrated into the genome and is expressed and for 2) subcellular localization. If GFP:SPS is expressed solely on the PM then the disrupted gene was presumably not essential for palmitoylation of that specific SPS. Potential outcomes for such experiments are depicted in Figure 4. It is important to remember that palmitoylation alone can be sufficient to take a GFP:SPS the plasma membrane (Zacharias et al., 2002). For this reason, we expect that chaperones or other transport proteins/genes that might have been necessary for normal trafficking of the full-length protein from which the SPS was derived, to the plasma membrane, will not be among the "background" (i.e non-S-PATs) genes trapped.

One of the primary advantages of virus-mediated gene infection is that it integrates only a single copy per cell genome. This advantage, while making it much easier to identify the mutated gene, virtually ensures that only one of two potential alleles of a gene will be hit in an "ideal diploid" cell line. It is possible that eliminating one of two S-PAT alleles in an ideal diploid cell will be insufficient to cause total redistribution of the GFP:SPS from the PM to the cytoplasm. However using the exquisite sensitivity provided by HTM will increase the likelihood of detecting any small changes should they occur. Similarly, electroporation of reporter plasmid DNA into cells can be controlled to reduce the likelihood of multiple integrations, but this also reduces the already-slim chances of randomly hitting both alleles of a gene within the same cell line. Most cell lines have variable numbers of chromosomes, often not resembling the normal diploid state of the organism from which it was derived. Additional copies of chromosomes increases the potential copy number of particular genes of interest thereby potentially decreasing the likelihood of generating a recognizable mutant phenotype (Figure 4). CHO-K1 (ATCC# CCL-61) cells are stably hypodiploid, meaning they have fewer than the original allotment of chromosomes and no spurious chromosomal duplications, thereby biasing the system slightly in favor of seeing a phenotypic change in response to mutagenizing a single allele of a gene. Additionally, if necessary, it is possible to bias the system further toward a gross redistribution of reporter upon mutagenesis of a critical gene by using a haploid cell line where only a single allele for each gene is represented in the genome. A stable, haploid cell line created from an androgenetic haploid embryo of

the frog *Rana pipiens* exists and is available from ATCC (#CCCL-145, designation ICR-2A). The growth characteristics as reported by ATCC, adherent and fibroblast-like, are appropriate for culture and microscopy. S-palmitoylation is functionally conserved in the frog, *Xenopus* (Dudler & Gelb, 1996; Hofemeister et al., 2002; Reddy et al., 1991), therefore, it is also most likely to be intact in *Rana*. The proposed retrovirus system effectively infects *Xenopus* and therefore should also work for *Rana*. If partial redistribution from the PM to the cytoplasm occurs, the trapped lines of interest can be subjected to additional rounds of insertional mutagenesis in attempts to reduce further the PM localization by trapping or mutagenizing genes that may be compensating for the originally mutated gene. This type of strategy will add dimensionality to the signaling network structure for the pathways leading to S-palmitoylation or any other such network being examined. Finally, it is also possible for the mutagenic element to integrate into a gene, even one critical for S-palmitoylation, without disrupting the function of the final translated protein. Due to the functional nature of the screen, a stable cell line with such a mutation would not be chosen for further analysis.

IV. *Identification and Functional Characterization of the Trapped Genes*

We fully expect to identify genes that when mutagenized will inhibit the process of S-palmitoylation. It is also likely that many rounds of mutagenesis will be necessary to find candidate clonal lines as well as to understand to degree to which we have saturated the genome. 5'- and 3'-RACE methods (Frohman et al., 1988; Zambrowicz et al., 1997) will be used to identify trapped genes in cell lines that have the morphological hallmark of a mutagenized gene critical for S-palmitoylation. The vectors have been carefully designed so that unique sequence in the SA, SD and GFP:SPS can be used in combination with universal primers and adaptors and protocols that are standard in the lab (see letters of support) to rapidly and efficiently identify the locus of integration of the mutagenic reporters. Identification of the mutated gene by sequence analysis will allow us to predict, in most cases, a possible function for the gene. However, further analysis will be necessary to understand the role of the identified genes in the pathway leading to S-palmitoylation. We are likely to trap genes that are involved in the synthesis of required precursors as well as S-PATs. We expect the background, false-positive

clones to outnumber the clones in which S-PATS are trapped with a significant number of the false positives occurring due to fragmentation of the reporter construct. As mentioned above, it is possible that the reporters could be physically fractured during the integration event (Voss et al., 1998) giving rise to independent, fractional integrations. Therefore, all clonal lines reporting a positive result will be surveyed for expression of an un-fragmented reporter, GFP:SPS. Reverse transcription-PCR and/or PCR of genomic DNA using primers to the 5' end of GFP and the 3' end of the SPS are efficient ways to determine the integrity of the integrated reporter. This type of prescreening will quickly reduce the potential positive clones to a workable number. We expect that the number of critical genes identified to be in the tens, not hundreds or thousands. Among these, the most interesting will be singled out for more extensive characterization. See below.

The polyA-trap-style of gene trap vector will not trap genes without introns. However, this smaller genomic space will be explored using promoter-trap vectors either as a supplement to the information that we gathered using polyA traps or as a backup in case we don't find critical genes searching with the polyA traps. While this genomic space is smaller, it is possible that the family(s) of genes responsible for S-palmitoylation could all fall into the intronless category.

Additional Methods to Characterize the Role of Trapped Genes in S-Palmitoylation

Rescue of the Mutant Phenotype

After identifying a candidate, "critical", trapped gene, a "rescue" of the mutant phenotype (i.e., GFP:SPS in the cytoplasm) will be attempted by re-expressing the wild-type version of the mutated gene in the mutant cell line. This will require cloning the full-length cDNA of the mutated gene, putting it into a suitable expression vector such as pcDNA3 (Invitrogen) and reintroducing the gene into the mutant cell line. Successful rescue of the mutant phenotype, as observed by the GFP:SPS relocalizing back onto the PM, will provide additional functional information supporting the identity of a gene involved in S-palmitoylation of the substrate used in the initial screen. An inability to rescue the mutant phenotype would suggest that the full extent of mutagenic integrations was not properly characterized at which point, different mutagenized clones would be sought.

Cross-substrate Specificity Tests

It is possible that there are many S-PATs, each with unique substrate specificity. This idea will be tested by expressing different, contextually unique S-palmitoylation substrates fused to another color of fluorescent protein (e.g., monomeric red fluorescent protein (mRFP) (Campbell et al., 2002)) in clonal cell lines containing putative or known S-PAT mutations. The red fluorescence of mRFP is easy to separate spectrally from GFP and would give simultaneous readouts of the subcellular localization of the two SPSs. Determining the degree of overlap among the other SPSs, and the original GFP:SPS would provide important information aiding the prediction of the numbers of PATs and their specificity. Other SPSs Representatives from three of the four classes of SPSs as described in (Linder & Deschenes, 2003) will also be tested. Specifically, the C-terminal tail of CD151(residues 236-end)(Yang et al., 2002); the c-terminus of Rho (Zacharias et al., 2002); The N-terminus of GAP-43 (Zacharias et al., 2002). The fourth class is mitochondrial proteins. The experiments outlined in this proposal are not designed to determine the palmitoylation state of proteins localized to the mitochondria.

Increasing the morphological homogeneity of the reporter cell line

A potentially useful alternative approach that can be explored if necessary is to create a stable cell line constitutively expressing the GFP:SPS of choice and then performing gene-trapping on this cell line using G-418 resistance as the only marker for selection of mutagenic integrations. The phenotypic marker for integration of the construct into a gene relevant to S-palmitoylation would still be redistribution of the fluorescent marker from the PM to the cytoplasm. While this approach does not utilize the convenience of fluorescence as a marker for integration, preselecting a line stably, efficiently and correctly expressing the GFP:SPS could increase the ability to distinguish mutagenic events that are truly disruptive of proper S-palmitoylation.

Identification of the trapped genes by sequence analysis and database homology searches, combined with the direct tests of functionality described above will allow us to reconstruct, at least in part, the genes that are required to induce S-palmitoylation of a

protein. The identities of the genes should also make it possible to place them in a logical sequence that leads to S-palmitoylation.

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5

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modification and improvements within the spirit and scope of the invention as set forth in the following claims.

What is claimed:

1. A method for identifying a gene that modulates subcellular localization of a protein comprising:

a) contacting a haploid cell with an insertional mutagen,

b) detecting a change in the subcellular localization of the protein; and

c) identifying the gene into which the insertional mutagen had inserted,

wherein the gene is identified as a gene that modulates subcellular localization of the protein.

FUNCTIONAL GENOMICS AND GENE TRAPPING IN HAPLOID CELLS

Abstract of the Disclosure

The present invention provides methods and compositions for performing functional genomics and gene trapping using haploid cells, including haploid vertebrate cells. The present invention further provides methods for identifying genes involved in cellular signaling pathways.

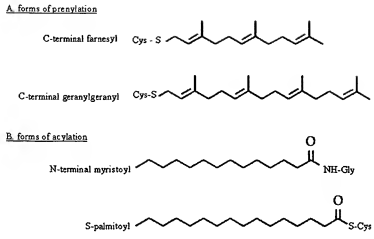
A. forms of prenylation
C-terminal farnesyl
Cys - S
C-terminal geranylgeranyl
Cys-S
B. forms of acylation
O
N-terminal myristoyl
NH-Gly
O
S-palmitoyl
S-Cys

Fig. 1

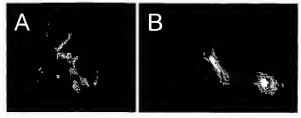

Fig. 2

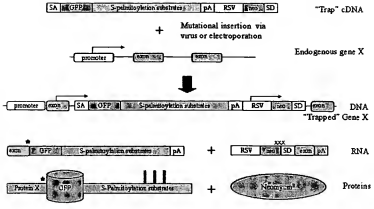
SA
GFP
pA
RSV
SD
"Trap" cDNA
+
Mutational insertion via
virus or electroporation
promoter
Endogeneous gene X
DNA
"Trapped" Gene X
exon
RSV
SD
RNA
Protein X
GFP
Neomycin
Proteins

Fig. 3

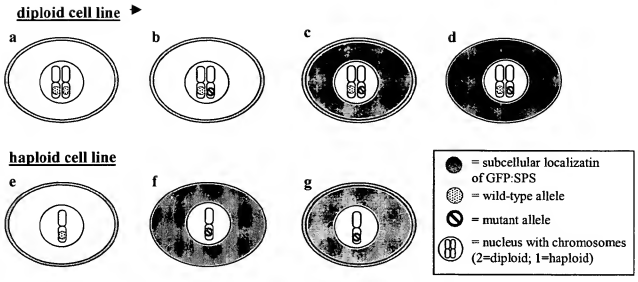
diploid cell line
a
b
c
d
haploid cell line
e
f
g
= subcellular localizatin of GFP:SPS
= wild-type allele
= mutant allele
= nucleus with chromosomes (2=diploid; 1=haploid)

Fig. 4